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Full Length Research Paper

Antihaemorrhagic Potential of *Citrullus colocynthis* Schrad (Cucurbitaceae) against *Naja naja karachiensis* (Black Pakistan Cobra) Venom

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Snake bites have been widely treated using plant remedies in rural areas of Pakistan. *Citrullus colocynthis* Schrad (Cucurbitaceae) is one of the plants used for the treatment of snake bite. This project evaluated the antivenom potential of the methanol extract from the fruit, pulp, seeds, and stems of *C. colocynthis* on a haemorrhage provoked by *Naja naja karachiensis* venom. A whole dried plant including fruit and stems of *C. colocynthis* was chopped and macerated to prepare methanol extract for anti-haemorrhagic testing. The reference haemorrhagic dose (RHD) and minimum effective neutralizing dose (MEND) values were determined for study extract. A dose dependent haemorrhagic response of venom was observed in a range of 0.1 to 4.0 µg/1.5 µL phosphate buffer saline (PBS) on the vitelline veins of fertilized chicken eggs (in their shells). The neutralizing response of *C. colocynthis* methanol extract against snake venom was valuable against venom haemorrhage and dose-dependent with a minimum effective neutralizing dose of 11 µg/1.5 µL of PBS. Methanol extract of *C. colocynthis* has antihaemorrhagic potential against *Naja naja karachiensis* venom. The present finding suggests that the methanol extract of *C. colocynthis* can be used to treat snake bites.

Key Words: Antiserum fertilized chicken eggs, maceration, reference haemorrhagic dose (RHD), minimum effective neutralizing dose (MEND).

INTRODUCTION

Snake bite poison is a worldwide work-related vulnerability. A large number of chronic physical handicap cases and numerous deaths especially in Pakistan are reported every year. Based on a previous study, mortality rate in Pakistan due to snake bite is about 20,000 per annum (Gutiée´rrez et al., 2006). The many poisonous snakes found in Pakistan are cobra (*Naja naja*) and krait (*Bangarus Caeruleus*). The snakes of the genus Naja are lethally venomous and are represented in Pakistan by two species: (i) Indian Cobra (*Naja naja naja*) and (ii) Central Asian (*Naja naja oxiana*). It has been reported previously that Black Pakistan Cobra (*Naja naja karachiensis*), a subspecies of *Naja naja oxiana*, and it is found in Southern regions of Pakistan (Warrell, 1999). Various pathologies, such as cytotoxicity, edema and myotoxicity, resulting from venom toxification of *Naja naja* (black cobra) (Chethankumar and Srinivas, 2008) are due to the presence of various enzymes in its venom which causes the degradation of key proteins in extracellular matrix (Baramova et al., 1990; Bjarnason and Fox, 1994), rigorous pain and skin necrosis (Forks, 1994). Previous studies have also elaborated haemorrhagic effect of venom which is attributed to the degradation of fibrinogen resulting in the inhibition of platelets aggregation (Ouyang et al., 1992).

Antisera/antibodies (obtained from equine animals) are considered most effective treatment for snake bite (Ouyang et al., 1992). However, antisera therapy leads to various rigorous unwanted effects such as pyrogenic

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reactions, anaphylactoid and late serum sickness. In addition, it is futile in defending venom-provoked haemorrhage, local tissue injury, necrosis and nephrotoxicity. Additionally, antisera production is a monotonous, expensive and lengthy practice which involves stringent storage condition (Chandrashekara et al., 2009; Mahadeswaraswamy et al., 2008).

Because of these problems in antiserum therapy, numerous medicinal plants have been proposed for the management of snake bites (Chopra et al., 1956). Pakistan possesses very large medicinal flora, and thus herbal remedies are very popular in rural areas (Khan et al., 2009). Herbal medications have also been adopted for the treatment of snake venom toxicity in different areas of Pakistan. The literature survey showed no systematic study of these plants to provide scientific grounds for their anti-venom potentials.

Citrullus colocynthis Schrad. (Cucurbitaceae) is one of the most popular plants which are locally utilized in Pakistan as a remedy for snake bite poisoning (Saeed, 1969). *C. colocynthis* is a spiny shrub found plentifully in arid calcareous rock areas in Pakistan (Saeed, 1969). Its various traditional uses are as: anticancer, hypertension, diabetes, antibacterial and to cure digestive problems (constipation) (Arena and Drew, 1980; Habs et al., 1984; Tannin-Spitz et al., 2007; Ziyyat et al.,1997; Brohi et al., 2003). Regardless of being widely studied for different bioactivities, no person has paid concentration to the scientific analysis of the anti-venom effects of *C. colocynthis* for which it is proposed as a folk medicine (Arena and Drew, 1980). Consequently, this present study was planned to assess the extract from *C. colocynthis* for its anti-venom activity to provide scientific grounds for its use as a traditional medicine for snake bite.

MATERIALS AND METHODS

Cobra snakes

Cobra snakes (*N. naja karachiensis*) were purchased from the Jogi colony of Haram gate (Baba-Shero Wala) Multan, Pakistan.

Snake venom antiserum reference

Lyophilized polyvalent snake venom antiserum was acquired from VINS Bio-products Ltd., India and was used as reference serum. Before utilization, the antiserum was reconstituted with normal saline.

Venom collection

The glands of the snake below its eyes was compressed in dark environment at room temperature (38℃) to collect venom, followed by lyophilization of venom. The lyophilized venom was then stored in an air-tight, sterilized, coloured, glass container at 4℃ until further use (Shaikh and Jokhio, 2006). Before use, venom was reconstituted in PBS and its concentration was used in terms of dry weight.

Plant materials

Whole dried plant including fruit and stems of *C. colocynthis* was procured from local market of Multan, Pakistan. Dr Mumtaz Bukhari, (plant taxonomist, Institute of pure and applied biology, Bahauddin Zakariya University, Multan, Pakistan) authenticated this plant material. Sample reference (number: Hass-02-*Citrullus colocynthis*) was registered in the Herbarium of Institute of pure and applied biology.

Preparation of plant extract, discs and eggs

The plant material was chopped and the weighed (500 g) quantity of ground material was soaked in methanol at room temperature for 4 weeks, to get extract. After the filtration of mixture, the filtrate was passed through evaporation step at 37℃ using a water bath. After 1 week of evaporation, the extract gotting weighed (38 g). Whatman number 2 filter was cut into discs (having 2 mm diameter) using hand punch (Sells et al., 1997).

Fertilized eggs of hen were acquired from big bird hatchery Multan, Pakistan. These eggs were incubated at 37.8°C/60% humidity using an incubator. After 4 days of incubation, the eggs (in their shells) were sprayed with 70% ethanol and dried in air for 10 min avoiding infectivity and rupturing of yolk (Sells, 2003).

Anti-haemorrhagic test

After four days of incubation period, the shells were removed from the wide part of the eggs. The previously prepared discs (having 2 mm diameter) were put on one of the vitelline veins of the egg embryo (Sells et al., 1997) followed by re-incubation of eggs for 3 h (Asuzu and Harvey, 2003). Various concentrations of venom in a range of 0.05 to 4.0 µg/1.5 µL of PBS were applied to the discs. Venom concentration, which created a haemorrhagic corona of 2 mm, was regarded as the reference haemorrhagic dose (RHD). A transparent ruler was used to measure the corona. Various concentrations of plant extract as well as antiserum were prepared by mixing them with RHD of venom in PBS and incubated at 37℃ for 30 min before appliance to the discs. The minimum concentration of antidote that is plant extract or reference antiserum, needed to eradicate haemorrhage was considered as the minimum effective neutralizing dose (MEND). Six replicates of antihaemorrhagic testing were conducted. In control trials, saline was employed as a substitute of venom or antidotes (Sells et al., 1997).

RESULTS AND DISCUSSION

A corona of 2 mm diameter was examined at 2.9 µg of venom/1.5 µL of PBS and RHD for venom from *N. naja karachiensis* was 2.9 µg/1.5 µL of PBS Figure 1. The *C. colocynthis* extract showed anti-haemorrhagic action at a concentration of ≥11 mg/1.5 µL of PBS and absolutely hampered the lesion development. Thus, 11 µg/1.5 µL of PBS was regarded as the MEND for the *C. colocynthis* extract and 0.05 µg/1.5 of µL PBS was MEND for reference antiserum (overall results are summarized in Table 1).

In this haemorrhagic test study, eggs were used in their shells which have useful aspects regarding time saving and low cost (cheap equipments) (Sells, 2003). It was observed that the haemorrhagic response of venom was

Table 1. Influence of *C. colocynthis* extract on corona provoked by venom from *N. naja karachiensis* on vitelline veins of egg embryos.

Venom/extract	Concentration of extract (µg/1.5 µL)	Corona diameter (mm)	Reduction from control (%)	MEND (µg/1.5 µL)	State of embryo after 3.5 h
N. naja karachiensis venom (2.9 µg/1.5 µL)	-	2	-	-	All alive
Extract + *N. naja karachiensis* venom (2.9 µg/1.5 µL)	5.5	<2 mm	<100	-	All alive
Extract + *N. naja karachiensis* venom (2.9 µg/1.5 µL)	11	0.0	100	11	All alive
Extract + *N. naja karachiensis* venom (2.9 µg/1.5 µL)	22	0.0	100	-	All alive

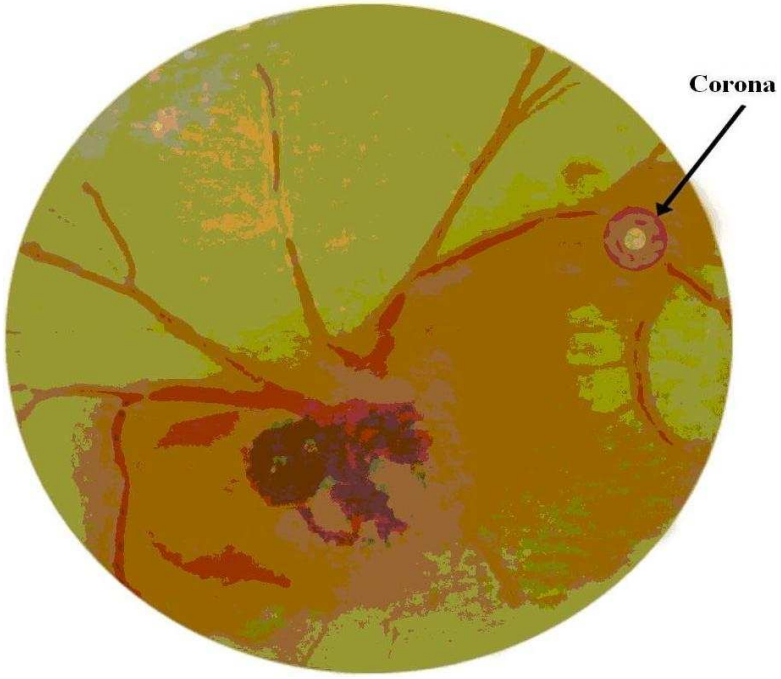

Figure 1. Corona (diameter 2 mm) development by venom from *N. naja karachiensis* on vitelline veins of egg embryos at 2.9 µg/1.5 µL phosphate buffer saline (reference heamorrhagic dose).

dose dependent exhibiting an augmenting trend of bleeding with the gradual increase in venom concentration from 0.1 to 4.0 µg/1.5 µL of PBS. No vein abrasions were detected at a concentration of 0.05 µg of venom/1.5 µL of PBS. However, venom with a concentration of 4.0 µg/1.5 µL of PBS and above proved fatal for the embryos. It was also observed that haemorrhagic activity of venom from *N. naja karachiensis* initiated from 0.1 µg/1.5 µL of PBS, consequently this concentration was regarded as its threshold value.

Complete elimination of corona was not observed with concentrations below 11 µg of plant extract/1.5 µL of PBS, however effective inhibition of corona development was assessed with a concentration of 11 µg of plant extract/1.5 µL PBS and higher. The results reveal that anti-haemorrhagic potential exhibited by plant extract was also dose dependent. No corona or lesion was seen when saline was used as a control.

In the present study, the value of MEND for *C. colocynthis* was 11 µg against the venom of *N. naja karachiensis*. This result elaborates that *C. colocynthis* has anti-venom potential as good as the other plants

narrated in the previous studies. For instance, *Calendula officinalis* exhibited MEND for *Echis ocellatus* venom at 30 mg, *Echis pyramidum* venom at 15 mg and *Echis leucogaster* venom at 10 mg (Sells et al., 1997), and the observed value of MEND for *Parkia biglobosa* was 5 mg/1.5 mL for *E. ocellatus* venom (Asuzu and Harvey, 2003). Consequently, present study demonstrated that *C. colocynthis* has potentials for healing in *N. naja karachiensis* envenomation. It also substantiated systematically the worth of the use of *C. colocynthis* in traditional medication for the cure of snake bite. Still, additional explorations are needed so as to isolate and recognize the bioactive ingredients from this plant, *C. colocynthis*.

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